METHODS OF DECALCIFICATION IN WHICH THE STRUCTURAL ELEMENTS ARE PRESERVED.

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For the purposes of investigating the soft structures of tissues containing lime salts, like bone, teeth, calcified cartilage, etc., it is necessary to remove the lime salts from the ground substance before thin sections can be made in the usual way; but in removing the lime salts there is danger of destroying or injuring the soft structures (cells and fibers of various kinds), and thus defeating the object in view.

Success depends largely upon first fixing and hardening the soft parts as for any tissue; secondly, it is necessary to use a decalcifier which would in no case injure the soft parts or which is restrained from injuring the soft parts by the addition of some chemical substance, which in brief may be called a *restrainer*.

Among all the decalcifiers, nitric acid ($H NO_3$) in various degrees of dilution has found perhaps the greatest favor. It has a powerful gelatinizing action even in dilute solutions and tends to deteriorate all soft structures in a short time if it acts alone or in aqueous solutions.

In 1888 it was discovered that the gelatinizing and softening action of nitric acid was almost wholly obviated by the use of a solution of alum (saturated aqueous solution of alum with the addition of 2 grams of chloral hydrate for each 100 cc. of alum solution). This alum solution, somewhat diluted as indicated below, acts also as a restrainer in decalcification with nitric acid.

The actual processes necessary for obtaining good sections are as follows:

1. The bone, tooth, or calcified cartilage is removed from the body as soon as possible after the death of the animal—that is, one proceeds exactly as for the soft tissues of the body and commences the fixing and hardening of the structural elements as soon as possible after death.

As the periosteum or perichondrium is an essential and integral part of a bone or cartilage, it should in no case be removed. The muscles may be cut away nearly to the bone, but not close enough to affect the periosteum or perichondrium. When this is done, if the object is a bone, it is broken with bone nippers or cut with a saw, so that the hardening agent can easily penetrate the medullary cavity or the spongy portion. The tissue is then put into 25 to 50 times its volume of the fixer and hardener. (One of the most satisfactory fixers and hardeners known to the writer is a mixture of 50 per cent. alcohol and picric acid, *i. e.*, 500 cc. water, 500 cc. 95 per cent. alcohol; picric acid, 2 grams.) The tissue is left one to three days in the picric alcohol, then one to three days in 67 per cent. alcohol, then one day or indefinitely in 82 per cent. alcohol.

2. DECALCIFICATION; (*Mixture A*).—Sixty-seven per cent. alcohol (*i. e.*, 95 per cent. alcohol, 2 parts; water, 1 part), 100 cc.; strong nitric acid, 3 cc. *In no case should the strong nitric acid and strong alcohol be mixed. An explosion would almost certainly follow.*

The bone or other calcified tissue is removed from the 82 per cent. alcohol and placed in the decalcifier. The smaller the volume of bone the more quickly will it become decalcified, other things being equal. The decalcifier is changed after 24 or 48 hours and a fresh supply added. If the bone is of considerable size it may be necessary to change the decalcifier two or three times. In all cases, too, the amount of decalcifier should be 25 to 50 times as great as the amount of tissue.

If the bone is small, as the radius or ulna of a kitten, the decalcification will ordinarily be complete in from three to five days. A stay of from 10 to 15 days seems not to injure the soft structures. It sometimes takes the longer time of 10 to 15 days to completely decalcify the skull of a salamander like Diemyctylus when none of the skin or soft parts are removed. One can determine when the decalcification is complete by inserting a fine needle or by slicing with a sharp scalpel. If any calcified matter remains, there will be a gritty feeling on inserting the needle or using the knife.

In this method the decalcifier is the nitric acid, and the restrainer is alcohol. The stronger the alcohol the more slowly the decalcification. A 3 per cent. aqueous solution of nitric acid would decalcify much more rapidly than when the alcohol is used, but the tissue would be far more liable to injury.

After a tissue is decalcified the decalcifier is poured off and the tissue rinsed a minute or so with water, then placed in 67 per cent.

alcohol one or two days, then in 82 per cent. alcohol until one is ready to make sections.

(*Method B.*) In this method the preliminary hardening and fixing are as for *Method A*, but the decalcifier is made as follows: A saturated aqueous solution of alum is diluted with an equal volume of water, and to each 100 cc. of this half-saturated alum solution 5 cc. of strong nitric acid is added.

The calcified tissue is taken from the 82 per cent. alcohol as before and placed in the decalcifier, and that changed every two or three days till the decalcification is complete. This will usually occur somewhat more quickly than with the mixture of alcohol and acid.

After the decalcification is complete the tissue is placed in running or in abundant water for a few minutes, and then placed in 67 per cent. alcohol one or two days, and then in 82 per cent. alcohol till one is ready to make sections.

The alum is the restrainer in this mixture. For teeth this is perhaps the better decalcifier. Both this and the mixture A preserve the soft structures, marrow cells, ciliated epithelium with the cilia, etc.

3. *Cutting the Sections.*—For cutting the sections one may remove from the 82 per cent. alcohol and cut free-hand. The paraffin method may also be used, but it renders the decalcified structures so hard that it is not satisfactory. The collodion method is, however, perfectly satisfactory, and is best applied in the following way: The bone or other decalcified tissue is dehydrated one day in 95 per cent. or stronger alcohol, then placed from four to five hours or longer in a mixture of equal parts of ether and alcohol. This is then poured off and thin collodion added. (Thin collodion, *i. e.*, equal parts of sulphuric ether and alcohol, 100 cc.; gun-cotton, 2 grams). After 5 to 24 hours or longer, if more convenient, the thin collodion is changed for thick. (Thick collodion, *i. e.*, equal parts of sulphuric ether and alcohol, 100 cc.; gun-cotton, 5 grams). After half a day or longer the tissue will be infiltrated with the thick collodion, and is to be imbedded as follows: A small paper box is made as for paraffin imbedding and the inside very lightly vaselined by rubbing vaselin on with the finger. Into this box the tissue is placed and successive layers of thick collodion poured upon it at intervals of fifteen or twenty seconds till the tissue is covered. The box is then either placed directly into a jar of *cold* 82 per cent. alcohol or for an hour or two into chloroform, and then into 82

per cent. alcohol. When the collodion is well hardened—*i. e.*, after a day or less—the sections may be cut. If one changes the 82 per cent. alcohol occasionally the tissues may remain months, and perhaps years, without deterioration.

For cutting the sections the paper box is removed, and then the imbedded object clamped directly in the microtome as a paraffin-imbedded object, or pieces of sheet cork may be used and the collodion-imbedded object put between these, and then clamped in the microtome. The sections are made with a well-wet knife and a drawing cut as usual.

4. *Staining and Mounting.*—The sections may be mounted in serial order on the slide, or if one does not care for that, they may be put into a watch-glass as cut and the thinnest selected for mounting.

For staining nothing has been found superior to the hematoxylin described on pp. 125–127 and eosin or picric acid. The specimens are mounted in balsam in the usual way or they may be mounted in glycerin or glycerin jelly.*

* For the different methods of decalcification proposed by others, see Lee. the Microtomist's Vade Mecum, 2d ed. For the use of alum as a restrainer for specimens acted upon by nitric acid, see Proceedings for 1889, Nos. 9 and 20 of Bibliography, pp. 42, 43.